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FEBS Letters 340 (1994) 241-244

FEBS 13744

FEBS LETTERS

Abundant expression of thromboxane synthase in rat macrophages

Yoshinori Tone[a], Atsuro Miyata[a], Shuntaro Hara[a], Susumu Yukawa[b], Tadashi Tanabe[a,*]

[a]*Department of Pharmacology, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan*
[b]*The 3rd Department of Internal Medicine, Wakayama Medical University, Wakayama 640, Japan*

Received 4 January 1994; revised version received 21 January 1994

Abstract

The cloned cDNA for rat thromboxane (TX) synthase with a size of 1851 bp contained a 1599-bp open reading frame which encoded a 533-amino acid protein sharing 79.7% identity with human TX synthase. RNA blot analysis was carried out with rat cells and tissues. Rat peritoneal macrophages most abundantly expressed mRNA for TX synthase, and its level was not changed by in vivo stimulation of casein. Bone marrow, spleen, lung and thymus also expressed the TX synthase gene. These findings suggest the possibility that TXA_2 plays a role in the immune system.

Key words: cDNA cloning; Thromboxane synthase; Gene expression; Macrophage; Rat

1. Introduction

Thromboxane (TX) A_2, a labile metabolite of arachidonic acid, promotes platelet aggregation and contracts vascular smooth muscle and glomerular mesangial cells [1,2], and has been implicated as a mediator in cardiovascular diseases, bronchial asthma, glomerulonephritis, etc. [3,4]. In the lesions of these diseases, macrophage infiltration is often observed [5,6], suggesting the involvement of macrophage-derived TXA_2 in the diseases. Recently, we succeeded in the cloning and expression of human platelet TX synthase cDNA, and demonstrated that the primary structure of the enzyme exhibited characteristics of cytochrome P450 [7,8]. We also found that the level of TX synthase mRNA in human erythroleukemia (HEL) cells was enhanced by phorbol ester [9]. For a better understanding of the pathogenesis of the diseases described above it is valuable to know about gene expression of TX synthase in macrophages. On the other hand, rats are frequently used as experimental models of cardiovascular diseases and glomerulonephritis. In this communication we report the cloning of rat TX synthase cDNA and the expression of the TX synthase gene in peritoneal macrophages, blood cells and tissues from rats.

*Corresponding author. Fax: (81) (6) 872 7485.

Abbreviations: TX, thromboxane; PCR, polymerase chain reaction; SD, Sprague–Dawley; bp, base pair(s); kb, kilobase pair(s).

2. Materials and methods

2.1. Molecular cloning and characterization of rat TX synthase cDNA

A rat megakaryocyte cDNA (λgt11) library was kindly provided by Dr. T. Doi (Osaka University). The probe for screening of the library was human platelet TX synthase cDNA (pHPTXS2 and pHPTXS6, [7]) radiolabeled with [α-^{32}P]dCTP (Amersham) by the random-priming method [10]. Screening of the library was performed according to standard protocols [11]. Hybridization was carried out overnight at 60°C and the membranes were washed at 55°C for 40 min in 1 × SSC (0.15 M NaCl and 15 mM sodium citrate, pH 7.0) containing 0.1% SDS. A positive clone (pRTXS1) was isolated and the cDNA insert excised by *Eco*RI digestion was ligated into pBluescript II SK(−). For polymerase chain reaction (PCR) cloning of the remaining 3′-region of the cDNA, the first strand cDNA was synthesized with 70 pmol of a dT_{17}-adaptor (5′-GACTCGAGTCGACTCGA$(T)_{17}$-3′) and the 1 μg of poly(A)$^+$ RNA prepared from peritoneal cells of Sprague–Dawley (SD) rats, which were intraperitoneally administered with peptone (3.75 mg/kg) 4 days before isolation. Amplification was performed with the adaptor primer and the P1 primer (5′-GACATGATCAAGGAGGTGTTGGTT-3′, nucleotide residues 280–304) synthesized according to the nucleotide sequence of the clone pRTXS1. The conditions for PCR amplification were as follows: denaturation at 94°C for 40 s, annealing at 55°C for 1 min and extension at 72°C for 3 min for a total of 30 cycles. After DNA blot analysis with the human TX synthase cDNA probe, the PCR product was purified and cloned into pBluescript II SK(−). The isolated clone pRTXS2-2 was ligated with the clone pRTXS1 at the *Bcl*I site to yield pRTXS3.

The DNA sequence was determined by the method of Sanger [12] using a BcaBEST DNA sequencing kit (Takara Shuzo Co.), or a Taq dye primer cycle sequencing kit on a model 373A DNA sequencer (Applied Biosystems Inc.). Computer analysis was performed with the aid of the SDC-GENETYX program supplied by Software Development Co. (Tokyo).

2.2. RNA blot analysis

The blood collected with EDTA from SD rats was centrifuged at 500 × *g* for 5 min at room temperature to separate the platelet-rich plasma and buffy coat from erythrocytes, and each of the two cell fractions were centrifuged at 1000 × *g* for 15 min [13]. Total RNAs of platelets and leukocytes were then isolated from each sediment. Peritoneal cells were isolated from 6- to 7-week-old male SD rats weighing about 200 g at day 1, 4 or 8 after an intraperitoneal injection of casein (0.75

SSDI 0014-5793(94)00129-J

mg/rat) in 15 ml of 0.9% NaCl [14]. The macrophage content in isolated peritoneal cells was estimated under a microscope by counting the number of cells stained by the Giemsa staining method. Total RNAs were isolated according to the acid guanidium thiocyanate procedure [15] and poly(A)$^+$ RNA was purified by oligo(dT)$_{30}$-latex (Nippon Roche). Total RNAs of rat tissues were prepared from normal 3- to 5-week-old male/female SD rats. 5 μg of poly(A)$^+$ RNA from each tissue and 20 μg of total RNA from blood cells and peritoneal cells were electrophoresed on a 1.0% agarose gel containing 1.5% formaldehyde, and transferred to a nylon membrane. Hybridization was carried out using rat ^{32}P-labeled TX synthase cDNA overnight as described in [9], and the membrane was washed at 65°C for 40 min in 0.1 × SSC containing 0.1% SDS.

3. Results and discussion

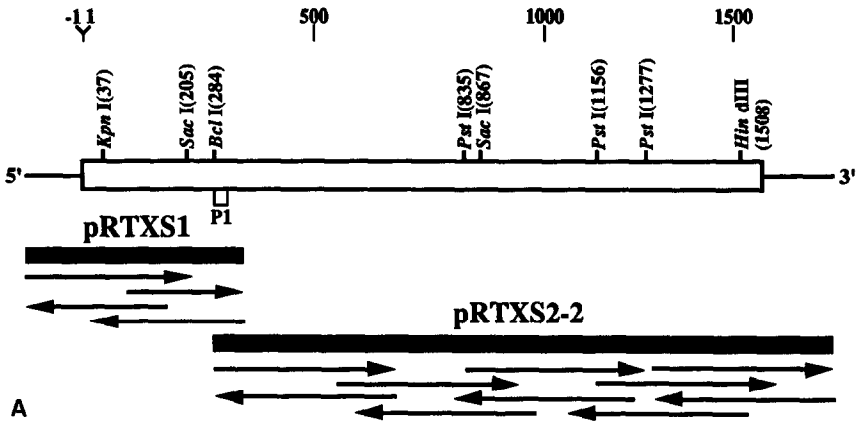
3.1. Cloning and sequencing of rat TX synthase cDNA

A TX synthase cDNA clone (pRTXS1) was isolated by screening of 3 × 10^5 clones of a rat megakaryocyte cDNA library (λgt11) with a human TX synthase cDNA as a probe. The isolated rat clone contained a 464-bp insert covering the 131-bp 5′-untranslated region and 333-bp protein-coding region. On RNA blot analysis using the pRTXS1 insert as a probe, the mRNA from rat peritoneal cells, mostly macrophages gave very intense signals. Therefore, the remaining 3′-downstream region of the TX synthase cDNA was amplified by the PCR technique using the cDNAs prepared with poly(A)$^+$ RNA from rat peritoneal cells. This amplified cDNA clone (pRTXS2–2) contained a 1536-bp insert including a polyadenylation motif AATAAA (nucleotides 1700–1705) and a poly(A)$_{23}$ tract. The inserts of the two cDNA clones totally covered a 1851-bp sequence of the cDNA for rat TX synthase (Fig. 1A). The full length of rat TX synthase cDNA contained a 1599-bp open reading frame encoding a 533-amino acid protein which has a calculated molecular weight of 59,974 Da (Fig. 1B). The result of the protein homology search demonstrated that rat TX synthase exhibited a 79.7% similarity with the human enzyme [7,16] and a 85.4% similarity with the mouse enzyme [17]. The amino acid sequence FGAGPRSCLG (residues 472–481) around the specific cysteine residue, which probably constitutes the fifth ligand of heme, was completely conserved among rat, human and mouse. The amino acid sequence from residue 77 to 87 of rat TX synthase was completely identical to that of human TX synthase, but was entirely different from that of mouse TX synthase, although the corresponding nucleotide sequences of rat and mouse enzymes showed a high similarity. The 3′-untranslated region of rat TX synthase cDNA was approximately 60 bp shorter than that of human and approximately 90 bp shorter than that of mouse.

3.2. Tissue expression of the rat TX synthase gene

Using a full-length rat TX synthase cDNA (pRTXS3) as a probe, RNA blot analysis was carried out to investigate the expression of TXS mRNA in rat blood cells and peritoneal cells. TX synthase mRNA was expressed in small amounts in platelets and leukocytes as shown in Fig. 2A. The result was different from a previous observation that a relatively high level of TX synthase mRNA was detected in human platelets [7]. However, the observed low level of TX synthase mRNA in rat platelets agreed with a low TX synthase activity determined in rat platelets [18]. A more detailed study is necessary to explain the different levels of TX synthase mRNA in human and rat. Interestingly, a large quantity of TX synthase mRNA was observed in rat peritoneal resident cells. We examined the effect of in vivo stimulation by casein on the mRNA level of peritoneal cells, because casein, as well as peptone, was effective to elicit macrophages. The mRNA level of TX synthase in peritoneal cells decreased one day after the intraperitoneal injection of casein, then increased to a level higher than that of resident cells during the following 3 days and went back to the level of resident cells 4 days later (Fig. 2A). On the other hand, the cellular population of macrophage was 70% in resident cells and decreased to 35% in peritoneal cells isolated one day after casein injection; most of the residual cells were polymorphonuclear neutrophils. However, the content of macrophage was returned to 95% during the following 3 days and slightly diminished to 80% 4 days later. Thus, it is likely that the change of the TX synthase mRNA level by casein corresponded to the relative cell population of macrophage in isolated peritoneal cells. Previously, Nüsing et al. [19] reported that neither TX synthase nor its enzyme activity could be demonstrated in purified human granulocytes. Furthermore, Tripp et al. [20] showed that TX synthase activity was unchanged in activated mouse peritoneal macrophages. These facts may suggest that TX synthase mRNA in peritoneal cells was mostly derived from peritoneal macrophages and the mRNA level in rat peritoneal macrophages did not change by stimulation with casein.

Fig. 2B shows the tissue specificity of the expression of the rat TX synthase gene. Only one band with a size of approximately 2.2 kb was observed by RNA blot analysis. During the course of the present study, Zhang et al. reported the cloning of mouse TX synthase cDNA and showed that the size of the transcript of the TX synthase gene in mouse kidney was about 100 to 150 bp shorter than those in lung and spleen [17]. Such a shorter transcript, however, was not found in rat kidney. Bone marrow was the organ richest in TX synthase mRNA followed by spleen, lung and thymus. The expression was also observed in liver, kidney and uterus, and a low but significant amount of the mRNA was seen in brain and heart. It is less possible that the TX synthase mRNA detected in brain and heart were caused by the contamination of blood cells, because they contained little TX synthase mRNA (Fig. 2A). The tissue distribution of rat TX synthase mRNA almost agreed with those of the

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                                                    5'--- GGATGGGATGTTTGCTCATTACATATTCCTTCCTTCACT   -91
GCCTACACACAGCTCAGGACTCCTATAAGGGCAGGACACGCTGATCAACTCTCCTCCTAGGAAGAACCCCTCTGGGGTCTCAGAGGAAAA    -1

ATGGAAGTGTTGGGGCTTCTCAAGTTCGAAGTCAGTGGTACCGTAGTGACTGTGACTCTGTCCGTGGTTCTCTTGGCCCTCCTGAAATGG     90
M  E  V  L  G  L  L  K  F  E  V  S  G  T  V  V  T  V  T  L  S  V  V  L  L  A  L  L  K  W      30

TACTCCACATCAGCGTTCTCAAGACTGAGGAAGTTGGGCATCAGACACCCTGAGCCTTCTCCTTTTGTTGGAAACTTGATGTTTTTCCGC    180
Y  S  T  S  A  F  S  R  L  R  K  L  G  I  R  H  P  E  P  S  P  F  V  G  N  L  M  F  F  R      60

CAGGGCTTTTGGGAGAGCCATTTGGAGCTCCGAGAGCGATATGGGCCTCTGTGTGGGTACTATCTTGGCCGTCGGATGTACATTGTCATT    270
Q  G  F  W  E  S  H  L  E  L  R  E  R  Y  G  P  L  C  G  Y  Y  L  G  R  R  M  Y  I  V  I      90

TCAGACCCAGACATGATCAAGGAGGTGTTGGTTGAGAACTTCAGTAACTTTTCCAACAGAATGGCCTCAGGTCTGGAACCCAAGCTGATA    360
S  D  P  D  M  I  K  E  V  L  V  E  N  F  S  N  F  S  N  R  M  A  S  G  L  E  P  K  L  I      120

GCAGACAGTGTCCTGATGTTACGTGACAGAAGATGGGAGGAAGTCAGAGGTGCCCTGATGTCTGCATTCAGTCCTGAAAAATTGAACGAG    450
A  D  S  V  L  M  L  R  D  R  R  W  E  E  V  R  G  A  L  M  S  A  F  S  P  E  K  L  N  E      150

ATGACACCTCTCATCAGCCAAGCCTGTGAACTTCTCCTGTCTCACTTAAAACACTCTGCGGCATCCGGGGACGCATTCGACATCCAGAGG    540
M  T  P  L  I  S  Q  A  C  E  L  L  L  S  H  L  K  H  S  A  A  S  G  D  A  F  D  I  Q  R      180

TGTTACTGCTGTTTTACCACAAATGTGGTGGCCAGTGTGGCCTTTGGCATCGAGGTGAACTCCCAGGATGCTCCAGAAGACCCCTTTGTG    630
C  Y  C  C  F  T  T  N  V  V  A  S  V  A  F  G  I  E  V  N  S  Q  D  A  P  E  D  P  F  V      210

CAACACTGCCAGCGTGTTTTTGCTTTCAGTACCCCAAGGCCTCTCCTGGCTTTAATTTTATCATTTCCATCCATAATGGTCCCATTGGCC    720
Q  H  C  Q  R  V  F  A  F  S  T  P  R  P  L  L  A  L  I  L  S  F  P  S  I  M  V  P  L  A      240

CGGATTCTGCCCAATAAGAACCGAGATGAACTGAATGGCTTTTTTAACACACTCATTAGGAATGTGATTGCCTTACGGGACAAGCAAACA    810
R  I  L  P  N  K  N  R  D  E  L  N  G  F  F  N  T  L  I  R  N  V  I  A  L  R  D  K  Q  T      270

GCAGAAGAGAGGCGGGGAGACTTCCTGCAGATGGTGCTGGATGCCCAGCGTTCCATGAGCTCTGTGGGTGTAGAAGCCTTTGACATGGTC    900
A  E  E  R  R  G  D  F  L  Q  M  V  L  D  A  Q  R  S  M  S  S  V  G  V  E  A  F  D  M  V      300

ACAGAAGCCCTGTCCTCTGCTGAGTGCATGGGGGACCCACCCCAAAGGTGTCATCCTACATCCACAGCTAAGCCTTTAACTGTGGATGAG    990
T  E  A  L  S  S  A  E  C  M  G  D  P  P  Q  R  C  H  P  T  S  T  A  K  P  L  T  V  D  E      330

ATTGCCGGCCAGGCTTTCCTCTTCCTCATCGCTGGCCACGAGATCACCACAAACACGCTCTCCTTCATCACATACCTGCTGGCCACCCAC   1080
I  A  G  Q  A  F  L  F  L  I  A  G  H  E  I  T  T  N  T  L  S  F  I  T  Y  L  L  A  T  H      360

CCTGAGTGCCAGGAGAGGCTTCTGAAAGAGGTGGACCTCTTCATGGAGAAGCATCCAGCCCCTGAGTACTGCAACCTGCAGGAAGGTCTG   1170
P  E  C  Q  E  R  L  L  K  E  V  D  L  F  M  E  K  H  P  A  P  E  Y  C  N  L  Q  E  G  L      390

CCCTATCTGGACATGGTGGTTGCAGAGACCCTGAGGATGTACCCACCAGCTTTCAGATTCACAAGGGAGGCAGCACAGGACTGTGAGGTG   1260
P  Y  L  D  M  V  V  A  E  T  L  R  M  Y  P  P  A  F  R  F  T  R  E  A  A  Q  D  C  E  V      420

CTGGGACAGCACATCCCTGCAGGTTCTGTGCTGGAGATTGCTGTGGGTGCCCTACACCATGACCCAGAGCACTGGCCGAACCCTGAGACC   1350
L  G  Q  H  I  P  A  G  S  V  L  E  I  A  V  G  A  L  H  H  D  P  E  H  W  P  N  P  E  T      450

TTTGACCCTGAAAGGTTTACAGCAGAAGCCCGGCTTCAGCAGAAGCCATTCACATACCTGCCTTTTGGAGCTGGTCCCAGGAGCTGCCTT   1440
F  D  P  E  R  F  T  A  E  A  R  L  Q  Q  K  P  F  T  Y  L  P  F  G  A  G  P  R  S  C  L      480

GGAGTACGGCTGGGGCTGCTGGTGGTCAAGCTGACACTGCTCCAGGTCCTACACAAGTTCCGCTTTGAAGCTTGCCCTGAGACTCAGGTT   1530
G  V  R  L  G  L  L  V  V  K  L  T  L  L  Q  V  L  H  K  F  R  F  E  A  C  P  E  T  Q  V      510

CCACTTCAGCTAGAATCCAAATCTGCTCTATGCCCCAAAAATGGAGTCTACGTCAAGATTGTGTCACGCTGACATAGAAGGCAGGTGTGA   1620
P  L  Q  L  E  S  K  S  A  L  C  P  K  N  G  V  Y  V  K  I  V  S  R  *                         533

B  AAGAGGAAGCACTTTGTAATAGTGACGTCTGTGCATCACTGTTAGGAGGGCGCCATTTATGTCACAGTTCTAAATGTTGAATAAATGTTT   1710
   GTCATGCTTC ---3'                                                                              1720
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Fig. 1. Rat thromboxane synthase. (A) Restriction map of cDNA encoding rat TX synthase. pRTXS1 was obtained by screening a megakaryocyte library and pRTXS2-2 was obtained by PCR as described in section 2. Only the relevant restriction sites are shown in this map. The protein-coding region is indicated by an open box, and the primer used for PCR is designated by P1. The direction and extent of sequencing are shown by arrows. (B) The nucleotide sequence of rat TX synthase cDNA and the deduced amino acid sequence of the enzyme. Nucleotides are numbered starting from the first nucleotide A of the translational initiation codon ATG, and amino acid residues are numbered starting from N-terminal methionine of the deduced primary structure of the rat enzyme. The underlined decapeptide indicates the conserved putative heme-binding site. The polyadenylation signal AATAAA is indicated by double underline. The poly(A) tract is not shown here.

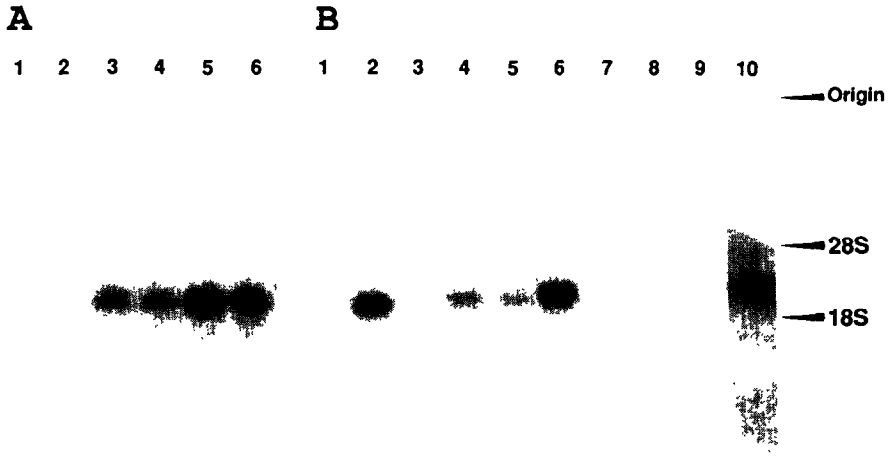

Fig. 2. Expression of the rat TX synthase gene. (A) RNA blot analysis of total RNAs (20 μg/lane) from blood cells and peritoneal cells: lane 1, platelets; lane 2, leukocytes; lane 3, resident peritoneal cells; lane 4, day 1 peritoneal cells post casein treatment; lane 5, day 4 peritoneal cells post casein treatment; lane 6, day 8 peritoneal cells post casein treatment. (B) RNA blot analysis with poly(A)$^+$ RNA (5 μg/lane) from rat tissues: lane 1, brain; lane 2, lung; lane 3, heart; lane 4, thymus; lane 5, liver; lane 6, spleen; lane 7, kidney; lane 8, testis; lane 9, uterus; lane 10, bone marrow.

human TX synthase enzyme protein and the specific enzyme activity [19], and was essentially identical with that of mouse TX mRNA [17]. The high levels of the expression of the TX synthase gene in spleen and thymus may reflect the mRNA of macrophages and dendric cells, because those cells in human were reported to have a TX synthase activity as high as platelets [19]. Recently, Namba et al. reported that mouse thymus and spleen were the organs rich in the TXA_2 receptor expression and suggested that the TXA_2 receptor is involved in and modulates immune responses [21]. In this context, our result that TX synthase mRNA is highly expressed in rat thymus and spleen, strongly supports the idea that TXA_2 plays a role in the immune system.

Acknowledgements: We thank Ms. T. Sugimoto, S. Bando and R. Tsuchiya for their technical assistance. This work has been supported in part by grants from the Ministry of Health, and Welfare and the Ministry of Education, Science and Culture of Japan and by Ciba-Geigy Foundation (Japan) for Promotion of Science.

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